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A PILOT SCALE PROCESS FOR THE PRODUCTION OF HIGH SHELF LIFE MULTI-FUNCTIONAL LIQUID BIOFERTILIZERS

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ABSTRACT

The mass production of liquid biofertilizers on the industrial scale with high cell viability and shelf life is still in infancy. Cost effective pilot scale (50l) process developed at purti enterprises, nagpur exhibits a high viability for azotobactor (1 x 1015), acetobactor (1 x 1015), psudeomonas (1 x 1018), frateuria (1 x 1017), bacillus (1 x 1017), trichoderma (2 x 109). The liquid biofertilizers were kept at room (28 ± 2°c) for shelf-life study. Liquid biofertilizers showed a significantly high viable count after storage of one to one and half year.

KEYWORDS: Alternative Agriculture, High Cell Viability, High Shelf Life & Liquid Bio Fertilizers

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Original Article

INTRODUCTION

Liquid Bio fertilizers

The liquid biofertilizers are believed to be the best alternative to synthetic fertilizers and conventional carrier based biofertilizers in the modern agriculture due to their high moisture retaining ability, longer shelf life than carrier based biofertilizers, better survival on the seed and nodulation, ease of handling, storage and transportation all favouring sustainable agricultural system of high productivity. They are the microbial preparations containing specific beneficial microorganisms which are capable of fixing or solubilizing or mobilizing plant nutrients by their biological activity (Pindi & Satyanarayana, 2012)..

Mass Production of High Cell Viability

The agricultural benefits possible from the use of selected liquid biofertilizers can be realised only when farmers obtain high quality inoculants on their legume seeds or soil before planting. Technology on growing rhizobia, preparing inoculants with suitable carrier materials, and distributing viable inoculants to farmers is essential. The most important constraint for adoption of liquid bio fertilizers was identified as the number of viable cells in the product. The production process of the inoculum is key to a final high-quality product (Bashan et al. 2014), since there is a direct relationship between the population density of mother culture and the quality of the final products (Stephens and Rask 2000). Hence, the increasing need for environmental friendly agricultural practices is driving the manufacturer to develop processes with high cell viability using the low cost medium. Related to the number of inoculant cells in carriers, the growing medium used to cultivate the microbial cells plays a very important role. For mass production of microbial cells of inoculant, the medium should be able to support

fast growth with high number of microbial cells of inoculant but the price of medium should be reasonably cheap. To our knowledge, there are no papers that have addressed the scale-up aspects of any fermentation process. Although there are few publications which address some aspects of the fermentation such as culture medium components (Onishi and Yokozeki 1996; Somashekar and Joseph 2000; Calventeet al. 2001) as well as batch, fed-batch or co-cultures to produce carotenoid pigments (Bhosale and Gadre 2001; Buzzini 2001; Govindaswamy et al. 2001; Wang et al. 2002) in such papers, the fermentation step was carried out in shake flasks and/or in small bench top fermentors (not larger than 1l) and neither the aspects of the scale-up of the process have been considered nor documented.

Shelf Life Studies of Liquid Inoculants

One of the main problems in inoculants technology is the survival of micro-organisms during storage and several parameters such as a culture medium, the physiological state of the microorganisms when harvested the process of dehydrates, the rate of drying the temperature storage and water activity (Aw) of the inoculums have an influence on their shelf life. So, studies to increase the shelf life of inoculants or finding alternate formations for carrier based inoculants are important.

Technical Ingredients (Organisms)

Azotobacter sp (Nitrogen Fixing)

Azotobacters are gram-negative, free living, polymorphic, non-symbiotic, aerobic diazotrophs. Its main role is to fix atmospheric nitrogen into the soil, however they releases vitamins, acetic acid, gibberellins, naphthalene, and other substances that inhibit certain root pathogens and improves root growth and uptake of plant nutrients (Mohapatra et. al; 2013). It is reported that it produces Polysaccharides (EPS) that protect against desiccation, mechanical stress, phagocytosis and page attack and participate in the update of metal ions as adhesive agents (Moulder and Brontonegoro 1974).

Acetobactor sp (Nitrogen Fixing)

Acetobacter, are gram negative, aerobic, non-symbiotic bacteria mainly associated with sugarcane, sweet potato and sweet sorghum, coffee plants. Owing to their unique metabolism, they are mainly involved in nitrogen fixation aerobically. They also produces growth promoting substances as IAA (Indole Acetic Acid) and GA (Gibberllic acid) that promote root proliferation and increase the number of rootlets, resulting in uptake of mineral, phosphate solubilisation and water which promote cane growth and sugar recovery in the cane.

Pseudomonas sp (Phosphate Solubilizing)

Pseudomonas are gram-negative, rod shaped, aerobic bacteria mainly associated with the supply of phosphorus through solubilization. Agricultural soils have large amounts of inorganic and organic phosphates; most of these are immobilised and unavailable to plants have been reported to have the ability to solubilize such insoluble inorganic phosphate compounds. Pseudomonas species have the ability to solubilise immobilised P in soil into bioavailable forms for plant uptake, taking part in the soil through a phosphorous cycle (Richardson et al., 2009). These bacteria, generally, improve the plant growth through direct effects on plants by producing plant growth promoting substances (Gaskins et. al. 1985, Lynch, 1982) by increasing the availability and uptake of mineral nutrients (Gaskins et al., 1985, Barber 1978, Barber et al.,1977) and by suppressing the soil-borne pathogens or other deleterious rhizosphere microorganisms (Gaskins et al., 1985, Schroth and Hancock 1982).

Frateuria sp (Potassium mobilizing)

The microbe, Frateuria aurentia is a beneficial bacterium capable of mobilizing available Potash into near the roots of the plants. It works well in all types of soil especially, low K content soil. It solubilizes the potash locked up in the soil into a simpler form and makes it available directly to the plants, helps to prevent soil erosion and improves the soil texture and fertility, helps to enrich soil micro-flora.

Trichoderma sp. (Plant Growth Promoting Bacteria)

Trichoderma sp. is a well-known plant growth-promoting fungi (Masunaka et al., 2011) that enhance plant nutrient uptake, production of growth hormones, and protect plants from pathogen infection (De Souza et al., 2008; Contreras-Cornejo et at., 2009; Zhang et al., 2014a), improves plant tolerance to environmental stress, and mineral absorption (Gupta et. al; 2014), seed germination rate, when applied for seed treatment and effective biofertilizer against many pathogenic fungi.

MATERIALS & METHODS

Materials

Microorganisms

Pseudomonas sp, Azotobactor sp, Acetobactor Sp, Trateuria sp, Bacillus sp, Trichoderma sp, were used throughout this work.

Maintenance of Cultures

Nutrient agar mentioned in the Table 1 was homogenized and then poured into well washed bijou bottles and tightly closed. They were sterilized in the autoclave at 121 ^{0}C for 15 min and allowed to solidify. The pure culture of *Azetobacter, Acetobacter, Psudeomonas, Bacillus, Frateuria* and *Trichoderma* obtained by isolation and purification processes were grown on nutrient agar media in petri dish at 30°C for 48 hrs. *Trichoderma* cultures were grown at 25-30 ^{0}C for 4-5 days.

Table 1: Media Composition (gL-1) for Production of Microbial Inoculants

Azotobacter sp		**Acetobacter sp**		**Psudeomonas & Bacillus**		**Frateuria sp**		**Trichoderma sp**	
Jensen's Medium				**Pikovskaya's Medium**		**Aleksendroy's Medium**			
Mannitol	10.0	Yeast Extract	10	Yeast Extract	0.5	$CaCO_3$	0.1	Potatose	200
KCl	0.2	$CaCO_3$	10	Dextrose	10	$AlKO_6SiO_2$	2	Dextrose	20
$MgSO_4$	0.1	Glucose	3	$(NH_4)_2SO_4$	0.5	$MgSO_4$	0.5		
$MnSO_4$	Trace			$Ca_3(PO4)_2$	5	Glucose	5		
$FeSO_4$	Trace			KCl	0.2	$Ca_3(PO4)_2$	2		
				$MgSO_{4,}.7H_20$	0.1	$FeCl_3$	Trace		
				$MnSO_4$	Trace				
				$FeSO_4$	Trace				
Temp (^{0}C)	30		25		25, 37		26		25
pH	7.2		7.4		7.1, 7,4		7.0		7.2

Preparation of Inoculum

A loopful of culture from nutrient agar slant is transferred to 5 mL of sterile nutrient broth and incubated at 37 ^{0}C for 24 hrs at 250 rpm. 20% of the grown cultures were transferred to 240mL sterile nutrient medium in 500 mL conical

flask. The medium was kept for incubation on a rotary incubator shaker at 250 rpm, 37 ^{0}C for 3-7 days. The inoculum was then subjected to serial dilution and platting to determine cell count and contamination.

Preparation of Mother Culture

After testing of inoculum culture, the culture, then processes for the preparation of the mother culture. 4750 ml of nutrient broth then inoculated with the inoculum culture and incubated aerobically on a rotary shaker at 250 rpm at 37 ºC for 24 hrs.

Analytical Methods

Gram Staining

The cell shape and Gram's property were examined after staining with the standard Gram staining procedure. A thin smear of bacterial isolate was prepared on the glass slide, air-dried and heat-fixed. It was stained in the following sequential order: covered with crystal violet for 30 s, washed with distilled water, covered with Gram's iodine solution for 60 s, washed with 95 % ethyl alcohol, washed with distilled water, counter- stained with safranin for 30 s and finally washed with distilled water. The stained and air-dried slides were examined under microscope using oil-immersion objective technique. Gram-positive bacteria retain the colour of crystal violet and stain with purple colour, while the Gram-negative took the colour of counter stain safranin appeared pink in colour.

Motility Test

This test was done using the hanging drop method. A drop of the test organisms in a saline suspension was placed on a cover slip. The cover slip was inverted and placed on a cavity slide, this was viewed under the microscope; a sharp darting movement in different directions across the field of view of the microscope indicated a positive result motility and showed that the organism had locomotive apparatus like flagella on that they can move

IMVIC Test

Indole Test

The tryptophan broth was inoculated with the test sample and incubated at 37 ^{0}C for 28 hrs. 0.5 ml of the Kovac's reagent was added and gently agitated and examined after 1 min. The upper layer of the liquid in the test tube turned red, indicating a positive result.

Methyl Red-Voges Proskaeu Test

In methyl red test, glucose 0.5 g, peptone water 1.5 g and di-potassium hydrogen phosphate 0.5 g were added into 100 ml of distilled water. The medium was sterilised for 15 min at 121^{0}C. After cooling it was inoculated and incubated for 3 days. Methyl red drops were in the flasks. Red colour showed a positive result for methyl red.

Citrate Utilisation Test

It was performed to see if the bacteria have the ability to utilise citrate as a sole source of carbon and energy for growth. An inoculum from a pure culture is transferred aseptically to a sterile tube of Simmons citrate agar. The inoculated tube is incubated at 35-37 ^{0}C for 24 hours. The growth on the slant and a change from green to blue in the medium indicates a positive test for growth using citrate.

Plate Counting Technique

The Nfb medium was prepared, sterilized and plated in sterile petriplates. The plates were kept at room temperature for 48 h. Eight equal sectors on the outside bottom of the petridishes were radially marked. Four sectors were used for replication of one dilution and four for another, allowing two dilutions per plate. Serial dilutions were prepared by transfer of 1 ml each of inoculum into 9 ml sterile water blanks to get 10^{-1} dilutions. Similarly, the dilutions were made serially upto 10^{-10}. From the dilutions, 5 µl was pipetted out and placed on the respective quadrant in the Petri plate. The plates were incubated at 28 ± 2°C without any disturbance and individual colonies were counted through this drop plate method (Somasegaran and Hoben 1994).

Shelf Life Studies

Bio-formulations of selected biofertilizer were obtained by mixing broth culture. These were packed in high density polythene bottle and stored at 28±2C and room temperature. The shelf life of the formulations was studied by drawing samples at regular interval of 30 days up to nine 18 months from date of mixing and the colony forming unit (cfu) was counted by serial dilution agar plate method.

PILOT PLANT BIOFERTILIZER PRODUCTION PROCESS

Pilot scale submerged cultures were carried out in batch, in polypropylene tank (maximum working volume of 50L) with Rushton agitator. The basic design is based on standard dimensions: tank diameter 0.94m and tank height 1.88m, equipped with three Rushton impellers of 0.31m (impeller/tank diameter ratio = 1:3). Rushton impellers are discs of diameter 0.19m and blade height and width of 0.06 and 0.07 respectively. The tank was equipped with four baffles of 1/10 of the tank diameter. All fermentations were conducted at 37^0C using an agitation speed of 52rpm. The air was spared on the bottom of the tank by a ring sparser at 0.5vvm and the head pressure was controlled at 0.2 kg/$cm^{2.}$ The pH at 7.0 ±0.2 was made by an on-off control, adding concentrated H_3PO_4. Filling volume, dissolved oxygen tension, temperature (culture and sterilisation), agitation and pH were displayed online and stored in a hard drive for further analysis using homemade acquisition control system.

Figure 1: Liquid Biofertilizer Pilot Plant

RESULTS & DISCUSSIONS

Biochemical Characterisation

The results of bio-chemical characteristics of isolated strains of bio-fertilizers are summarized in Table 2. The isolated bacterial strains for nitrogen fixing was rod shaped, Gram's negative and identified as *Azotobacter* & Acetobacter, which is well known as free living N_2 fixing bacteria. Based on the halo zone formation on Pikovkaya's

(PKV) agar plate by *Psudeomonas sp* and *Bacillus sp.* was considered as phosphate solubilizing bacteria. Gyaneshwar et al., 1999 also reported that the colonies with clear halo zones are considered to be PSB. Mobilization observed on Aleksendry's agar plate was considered as potash mobilising bacteria and identified as *Frateuria.*

Table 2: Biochemical Characterisation of Microorganisms

No.	Organism	Gram	pH	Appearance	Motility	Viable Cell Count (cfu/ml)
1	*Azotobactor*	-ve	7.0 -7.5	Rod Shaped	Motile	1×10^{15}
2	*Pseudomonas*	-ve	7.0 -7.5	Rod Shaped	Motile	1×10^{19}
3	*Acetobactor*	-ve	7.0 -7.5	Rod Shaped	Motile	1×10^{18}
4	*Frateuria Sp.*	-ve	7.0 -7.5	Rod Shaped	Motile	1×10^{17}
5	*Bacillus Sp.*	+ve	7.0 -7.5	Rod Shaped	Motile	1×10^{17}

Pilot Scale Production

The pilot process development started by searching a low-cost production medium and establishing if the viable cells produced in pilot garments with this latter medium were as effective in antagonism as those produced in laboratory media in Petri plates or in 250 ml shaking flasks. In vitro assays showed that the culture medium and the scale did not affect the antagonistic capacity of the all microbial strains. Results were practically identical to those given in Table 2 regardless of the culture medium and the type and scale of bioreactor in which the microorganism was produced). This allows to continue the development of the liquid fermentation and the scale-up of the process up to a 50L pilot fermentor

Table 3: Pilot Scale Microbial Count

Sr. No	Name of Organism	Cell Count
1	*Azotobactor*	1×10^{15}
2	*Pseudomonas*	1×10^{18}
3	*Acetobactor*	1×10^{15}
4	*Frateuria Sp.*	1×10^{17}
5	*Bacillus Sp.*	1×10^{17}

Shelf Life

Liquid biofertilizers are usually prepared as liquid inoculants containing effective microorganisms. These identified strains were used for the preparation of bio-formulations using water and evaluated for their viable cell count during storage period of 540 days in laboratory conditions. A general decline in cfu count was noticed for liquid based bio-formulations. However, these bioformulations retained more than $10^{13\text{-}14}$ cfu/ml viable propagules even after 540 days except for Trichoderma. The cfg count is summarised in Table 4 for all bacterial strains.

Table 4: Shelf Life Study

No	Month	Acetobacter	Azotobacter	P. S. B	K. M. B.	Tricho
1	Nov-16	2×10^{18}	1×10^{19}	2×10^{19}	1×10^{18}	2×10^{9}
2	Dec-16	2×10^{18}	1×10^{19}	1×10^{19}	1×10^{18}	2×10^{9}
3	Jan-17	2×10^{18}	1×10^{19}	2×10^{18}	1×10^{18}	2×10^{9}
4	Feb-17	2×10^{18}	1×10^{19}	2×10^{18}	2×10^{17}	2×10^{9}
5	Mar-17	2×10^{16}	1×10^{18}	2×10^{18}	2×10^{17}	2×10^{9}
6	Apr-17	2×10^{16}	1×10^{18}	1×10^{17}	1×10^{17}	1×10^{9}
7	May-17	2×10^{16}	2×10^{17}	1×10^{17}	1×10^{17}	1×10^{9}
8	Jun-17	2×10^{16}	1×10^{17}	2×10^{16}	1×10^{17}	1×10^{9}
9	Jul-17	2×10^{16}	2×10^{15}	2×10^{16}	1×10^{17}	1×10^{8}

Table 4: Contd.,						
10	Aug-17	1×10^{15}	2×10^{15}	2×10^{16}	1×10^{17}	1×10^{8}
11	Sep-17	1×10^{15}	2×10^{15}	1×10^{16}	2×10^{16}	1×10^{8}
12	Oct-17	1×10^{15}	3×10^{14}	1×10^{16}	2×10^{16}	2×10^{7}
13	Nov-17	2×10^{14}	3×10^{14}	2×10^{15}	2×10^{15}	2×10^{6}
14	Dec-17	2×10^{14}	3×10^{14}	1×10^{15}	2×10^{15}	2×10^{6}
15	Jan-18	2×10^{14}	2×10^{14}	1×10^{15}	1×10^{15}	2×10^{6}
16	Feb-18	1×10^{14}	2×10^{14}	1×10^{15}	1×10^{15}	1×10^{6}
17	Mar-18	1×10^{14}	2×10^{14}	1×10^{15}	1×10^{15}	1×10^{6}
18	Apr-18	1×10^{14}	2×10^{14}	1×10^{15}	1×10^{15}	1×10^{6}
19	May-18	1×10^{14}	2×10^{14}	1×10^{15}	1×10^{15}	1×10^{6}

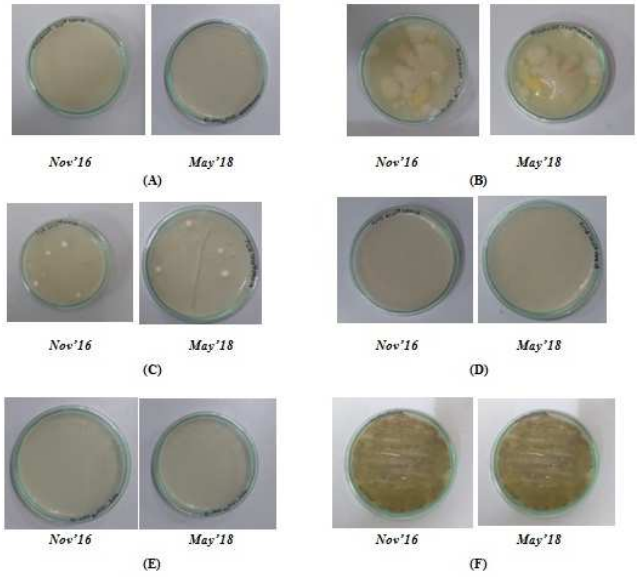

Figure 2: Microbial Count in Shelf Life Study for (1.5 yrs)
(a) Azotobacter, (b) Azotobacter,
(c) Potash Mobilising Bacteria, (d) NPK,
(e) Phosphate Solubilizing Bacteria (f) Trichoderma

(a) (b) (c) (d) (e)

Figure 3: Liquid Biofertilizers by Purti Enterprises, (a) Acetobacter, (b) Azotobacter, (c) Potash Mobilizing Bacteria, (d) NPK, (e) Phosphate Mobilizing Bacteria

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